# Prolyl Endopeptidase Inhibitors from *Syzygium samarangense* (Blume) Merr. & L. M. Perry

Evangeline C. Amor<sup>\*,a</sup>, Irene M. Villaseñor<sup>a</sup>, Amsha Yasin<sup>b</sup>, and M. Iqbal Choudhary<sup>b</sup>

- <sup>a</sup> Institute of Chemistry, College of Science, University of the Philippines, Diliman 1101 Quezon City, Philippines. Fax: (632)9205427. E-mail: evangeline.amor@up.edu.ph
- <sup>b</sup> International Center for Chemical Sciences at HEJ Research Institute of Chemistry, University of Karachi, Karachi 75270, Pakistan
- \* Author for correspondence and reprint requests
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Compounds isolated from the hexane extract of the leaves of Syzygium samarangense (Blume) Merr. & L. M. Perry were tested for inhibitory activity against the following serine proteases: trypsin, thrombin and prolyl endopeptidase. The compounds were identified as an intractable mixture of  $\alpha$ -carotene and  $\beta$ -carotene (1), lupeol (2), betulin (3), *epi*-betulinic acid (4), 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (5), 2'-hydroxy-4',6'-dimethoxy-3'-methylchalcone (6), 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (7), 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (7), 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (7), 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone (8) and 7-hydroxy-6'-methoxy-3',5'-dimethylflavanone (9). Hydrogenation of compounds 5, 6 and 7 yielded compound 8, 2'-hydroxy-4',6'-dimethoxy-3'-methyldihydrochalcone (10) and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyldihydrochalcone (10) and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyldihydrochalcone (11), respectively. The hydrogenated products of compounds 6 and 7 were also tested for enzyme inhibitory activity. In addition,  $\beta$ -sitosterol (12) and  $\beta$ -D-sitosterylglucoside (13) were also isolated. This is the first report of the isolation of compounds 1-6, 8 and 13 from this plant. Compounds 3-8 and 10 exhibited significant and selective inhibition against prolyl endopeptidase among three serine proteases. This is the first report of this kind of activity for all these compounds.

Key words: Syzygium samarangense, Prolyl Endopeptidase, Flavonoids

## Introduction

Syzygium samarangense (Blume) Merr. & L. M. Perry (Myrtaceae) is locally known in the Philippines as "makopa". The plant has been reported to have antibacterial (Santos, 1981), antidiabetic (Nonaka *et al.*, 1992) and immunostimulant (Srivastava *et al.*, 1995) activities. Compounds previously isolated from this plant include mearnsitrin, 2'-C-methyl-5'-O-galloylmyricetin-3-O- $\alpha$ -Lrhamnopyranoside, desmethoxymatteucinol (Nair *et al.*, 1999), 4',6'-dihydroxy-2'-methoxy-3',5'-dimethylchalcone, methyl 3-*epi*-betulinate, oleanolic acid, jacoumaric acid, ursolic acid, arjunolic acid (Srivastava *et al.*, 1995), samarangenin A and samarangenin B (Nonaka *et al.*, 1992).

Prolyl endopeptidase (PEP) is a post-proline cleaving enzyme (EC 3.4.21.26). It was first isolated from the human uterus as an oxytocin-inactivating enzyme, specifically cleaving the peptide bond at the carbonyl side of proline residues (Tezuka *et al.*, 1999). In the central nervous system, PEP has been playing an important role in maturation and degradation of proline containing peptide hormones and neuropeptides, which are suggested to participate in learning and memory processes such as vasopressin, substance P, thyrotropin-releasing hormone (TRH), angiotensin, LH-RH neurotensin, angiotensin II, oxytocin and bradykinin. Currently, new drugs are required that can improve memory and learning or delay the neurodegenerative process in conditions such as Alzheimer's disease. Inhibitors of prolyl endopeptidase may improve memory by blocking the metabolism of endogenous neuropeptides and have possible potential as antiamnesiac drugs (Tezuka et al., 1999; Yoshimoto et al., 1987). In fact, it has been hailed as a new class of memory enhancing drugs (De Nanteuil et al., 1998).

There are two known kinds of PEP inhibitors, peptidic inhibitors (De Nanteuil *et al.*, 1998; Feher *et al.*, 1999; Hermecz and Kanai, 2000) and non-peptidic inhibitors (Christner *et al.*, 1998; Lee *et al.*, 2000; Inamori *et al.*, 1997; Fan *et al.*, 1999, 2000, 2001). Peptidic inhibitors are known to contain an electrophilic center such as an *alpha*-keto

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*beta*-amide group and this group is considered to be an active center. Non-peptidic inhibitors, on the other hand, are further grouped into two types. One type contains either a catechol or pyrogallol group and the second type, none. In the former, the catechol or pyrogallol group is believed to be responsible for its PEP inhibition (Fan *et al.*, 1999). Most PEP inhibitors reported are synthetic and only a few of the natural inhibitors are from plant sources (Tezuka *et al.*, 1999). This study explores the PEP inhibitory activity of compounds from *S. samarangense*.

#### **Results and Discussion**

Several compounds were isolated from the hexane extract of the leaves of S. samarangense and their structures (Fig. 1) elucidated through spectral analyses and by comparison with the literature. These were identified as mixture of  $\alpha$ - and  $\beta$ -carotene (1), lupeol (2), betulin (3), *epi*-betulinic acid (4), 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (5), 2'-hydroxy-4',6'-dimethoxy-3'-methylchalcone (6), 2',4'-dihydroxy-6'-methoxy-3',5'dimethylchalcone (7), 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone (8) and 7-hydroxy-5methoxy-6,8-dimethylflavanone (9). Hydrogenation of compounds 5, 6 and 7 yielded compound 8, 2'-hydroxy-4',6'-dimethoxy-3'-methyldihydrochalcone (10) and 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyldihydrochalcone (11), respectively. In addition,  $\beta$ -sitosterol (12) and  $\beta$ -D-sitosterylglucoside (13) were also isolated. This is the first report of the isolation of compounds 1-6, 8 and 13 from this plant. The <sup>13</sup>C NMR chemical shifts for compound 5 are reported for the first time and those of compound 7 are also reported because of differences with that reported in the literature (Srivastava et al., 1995). These are summarized in Table I.

Among the compounds, compound **2** was previously reported to be a competitive inhibitor of trypsin and chymotrypsin with K<sub>i</sub> values of 22 and 8  $\mu$ M, respectively (Rajic *et al.*, 2000). In addition, compound **13** has been reported to exhibit PEP inhibitory activity with IC<sub>50</sub> of 27.5 ppm (Lee *et al.*, 1998).

Compounds 3-11 were then tested for inhibitory activities against three serine proteases trypsin, thrombin and prolyl endopeptidase as described previously (Rahman *et al.*, 2002). The results are summarized in Table II. All compounds except 9 showed significant inhibitory activity against PEP while compounds 3-10 did not exhibit significant inhibitory activity against thrombin and trypsin and therefore are specific inhibitors for PEP.

Flavonoids with PEP inhibitory activity have been reported. Of the 20 flavonoids tested containing the flavone, flavonol, flavanone and flavanol skeletons, luteolin and quercetin (flavones) exhibited potent PEP inhibitory activity with an  $IC_{50}$ of 0.17 and 0.19 ppm, respectively (Lee et al., 1998). In addition, flavans (Fan et al., 1999, 2001) were also reported to have PEP inhibitory activity. A catechol or pyrogallol moiety in ring C and a 7-OH group in flavones are believed to be responsible for the observed activity. The tested flavonoids in this research work having a chalcone (5, 6 and)7), dihydrochalcone (8, 10 and 11) and a flavanone (9) skeleton do not contain a catechol nor a pyrogallol moiety in ring C and are less potent than luteolin or quercetin. However, the inhibitory activities of the active compounds are comparable, if not greater than other natural non-peptidic inhibitors that contain a pyrogallol or catechol moiety. For example, 8 (IC<sub>50</sub> =  $12.5 \,\mu\text{M}$  or  $3.55 \,\text{ppm}$ ) contains no catechol moiety and yet is more active than orientin (IC<sub>50</sub> = 38.5 ppm), which has a catechol moiety (Lee et al., 1998). It is more active

Table I.  $^{13}$ C NMR chemical shift data of compound **5** and **7**.

С	Compound <b>5</b> in C <sub>3</sub> D <sub>6</sub> O at 125 MHz (ppm)	Compound <b>7</b> in CDCl <sub>3</sub> at 125 MHz (ppm)
$ \begin{array}{c} 1 \\ 2 \\ 3 \\ 4 \\ 5 \\ 6 \\ \beta \\ \alpha \\ C=O \\ 1' \\ 2' \\ 3' \end{array} $	$136.4 \\ 129.0 \\ 129.7 \\ 130.7 \\ 129.7 \\ 129.0 \\ 142.3 \\ 128.6 \\ 193.1 \\ 104.4 \\ 166.4 \\ 106.0$	135.3 128.9 128.4 130.2 128.4 128.9 142.9 126.7 193.4 106.6 162.0 109.0
4' 5' 6' 6'-OCH <sub>3</sub> 3'-CH <sub>3</sub> 5'-CH <sub>3</sub> 2'-OH 4'-OH	161.8 91.5 163.3 56.1 7.36	159.3 109.0 158.8 62.3 8.2 7.6 –



Fig. 1. Structures of compounds 2–11: lupeol (2); betulin (3); *epi*-betulinic acid (4); 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (5); 2',4'-dihydroxy-6'-methoxy-3'-methylchalcone (6); 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone (7); 2',4'-dihydroxy-6'-methoxy-3'-methyldihydrochalcone (8); 7-hydroxy-5-methoxy-6,8-dimethylflavanone (9); 2'-hydroxy-4',6'-dimethoxy-3'-methyldihydrochalcone (10); 2',4'-dihydroxy-6'-methoxy-3',5'-dimethyldihydrochalcone (11).

than gallic acid (IC<sub>50</sub> = 487  $\mu$ M), which contains a pyrogallol group (Fan *et al.*, 1999). It is also more potent than any of the inhibitors from *Salvia deserta* (Tezuka *et al.* 1999) whose IC<sub>50</sub> values range from 53.4 to > 1000  $\mu$ M, which are all examples of non-peptidic inhibitors from a plant source. It is even more potent than bacitracin (IC<sub>50</sub> = 129.26  $\mu$ M), the positive control and a peptidic inhibitor. Flavanones have so far been inactive against the PEP enzyme (Lee *et al.*, 1998; Fan *et al.*, 2000), which correlates with the observed negative result for compound **9**.

There is one chalcone, licuroside, reported as a PEP inhibitor with an  $IC_{50}$  value of  $26.5 \,\mu\text{M}$  (Fan *et al.*, 2000). In comparison, licuroside is more potent than **5**, **6** and **7** whereas **8** is more potent than licuroside. Comparing the PEP inhibitory activities of the isolated chalcones and dihydrochalcones (Table II), the results indicate that the loss

of unsaturation in the  $\alpha - \beta$  bond of compounds 5, 6 and 7 enhances inhibitory activity against PEP as seen in the increase in the IC<sub>50</sub> values of the corresponding hydrogenated compounds 8, 10 and 11.

Among the triterpenoids, **4** is the most potent PEP inhibitor followed by **2** and then **3**. **2** and **3** are structurally similar differing only in the degree of oxidation of C-28. It is apparent that the degree of oxidation is inversely proportional to their inhibitory activity. This suggests that lipophilic interactions are predominant for efficient enzyme-inhibitor binding interaction leading to an increase in inhibitory activity of **2** compared to **3**. **4** is different from **2** and **3** in the degree of oxidation of C-28 and the configuration of C-3-OH. Configurational changes in **4** involving C-3 appear to have led to more efficient enzyme-inhibitor binding interaction resulting in a significant increase in its

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Compound	% Inhibition (concentration [mм])		ІС <sub>50</sub> [μм]	Table II. Inhibitory activities of the compounds from <i>S. sa-</i> <i>marangense.</i>
	Trypsin	Thrombin	PEP	
1	_	_	<sup>a</sup> 64.4%	
2	$K_i = 22 \ \mu M^b$	49.2 (0.2)	$65.0 \pm 3.2$	
3	24.4 (0.125)	NA <sup>c</sup> (0.125)	$101.6 \pm 1.3$	
4	28.9 (0.1)	32.1 (0.1)	$14.8 \pm 0.4$	
5	5.6 (0.125)	NA <sup>c</sup> (0.125)	$37.5 \pm 1.0$	
6	15.8 (0.25)	30.7 (0.25)	> 200	
7	$NA^{c}(0.25)$	1.8 (0.25)	$149.8 \pm 7.1$	
8	31.9 (0.25)	14.9 (0.25)	$12.5 \pm 0.2$	
9	7.4 (0.1)	$NA^{c}(0.1)$	<sup>a</sup> 13.9%	
10	2.7 (0.5)	10.0(0.5)	$158.5 \pm 0.1$	
11	38.2 (0.25)	62.1 (0.25)	$98.3 \pm 0.8$	
Bacitracin	-	-	$129.26 \pm 3.28$	
(PEP positive control)				- Not performed in this as-
Leupeptin	$0.026 \pm 0.001^{d}$	-	-	say.
(Trypsin positive control)				<sup>a</sup> % Inhibition at 0.5 mM.
Leupeptin	-	$0.045 \pm 0.003^{d}$	-	<sup>c</sup> Rajic <i>et al.</i> , 2000.
(Thrombin positive control)				$^{d}$ IC <sub>50</sub> in $\mu$ M.

inhibitory activity. It is highly probable that **4** is interacting with a different site of the enzyme compared with **2** and **3**. The acidic moiety in **4** seems to have an important contribution towards the strong inhibitory activity possibly through H-

bonding interactions. This is the first report of PEP inhibitory activity for dihydrochalcones and triterpenoids with a lupane skeleton. All of the active isolated compounds fall under non-peptidic inhibitors with neither a catechol nor a pyrogallol moiety. Furthermore, this research work establishes the selectivity of compounds 3-8 and 10 against the prolyl endopeptidase enzyme.

# Experimental

## General

All the solvents used for extraction, isolation and purification were technical grade and were distilled before use. Normal phase column chromatography (NPCC) with gradient elution was the technique generally employed in the isolation of natural products using silica gel, type-60 (70–230 mesh, Merck). Thin layer chromatography (TLC) was used to monitor separation with GF-254 aluminum plates (Merck). TLC plates were visualized under UV lamp set at 254 and 365 nm. Spray reagent used was 65% CeSO<sub>4</sub>·H<sub>2</sub>SO<sub>4</sub>. FT-IR spectra were recorded with a JASCO A-302 IR spectrophotometer or a Bio-RAD FTS 40-A spectrophotometer. EI-MS was recorded with a Finnigan MAT 312 equipped with a Masspec Data system at 70 eV. The 1D and 2D NMR spectra were recorded with a Bruker AM 300 MHz (75 MHz for <sup>13</sup>C) FT NMR or Bruker AM 500 MHz (125 MHz for <sup>13</sup>C) spectrometer with Aspect 3000 and X-32 data and a JEOL Lambda 400 MHz (100 MHz for <sup>13</sup>C) NMR spectrometer. The melting points were recorded with a Yanaco micro melting point apparatus or a Fisher-Johns melting point apparatus. The recorded melting points were all uncorrected.

## Plant material

Leaves of the plant were sampled from Parañaque, Metro Manila. This was authenticated and a voucher specimen with accession No. 14258 was submitted to the Dr. Jose Vera Santos Herbarium, Institute of Biology, University of the Philippines in Diliman.

#### Extraction and isolation

The ground leaves of *S. samarangense* (2.9 kg) were extracted at room temperature with methanol and subsequently partitioned between  $H_2O/$  hexane (1:6 v/v), from which the hexane extract was obtained and concentrated (140 g oily residue). This was subjected to fractionation by NPCC on silica gel (1:10 m/m) employing gradient elution

(10% increments) with hexane, dichloromethane/ hexane, dichloromethane, methanol/dichloromethane and finally with methanol, giving fractions 1-4.

Compound 1 (20 mg) and compound 6 (75 mg) crystallized out of fraction 1, eluted with hexane and 10% dichloromethane/hexane. Compound 1 was purified by recrystallization in acetone while compound 6 was purified by recrystallization in 5% dichloromethane/hexane.

Sequential NPCC of fraction 2, eluted with 20% to 50% dichloromethane/hexane, on silica gel employing gradient elution (10% increments) with hexane and dichloromethane/hexane yielded compounds **7** (3 g), **2** (20 mg) and **12** (20 mg).

Sequential NPCC of fraction 3, eluted with 60% dichloromethane/hexane to 30% methanol/dichloromethane, on silica gel employing gradient elution (10% increments) with hexane, dichloromethane/hexane, dichloromethane, methanol/dichloromethane yielded compounds **3** (15 mg), **4** (30 mg), **5** (100 mg), **8** (3 mg), and **9** (50 mg).

Compound **13** (10 mg) crystallized out of fraction 4, eluted with 40% methanol/dichloromethane and methanol, was purified by recrystallization in methanol.

## 2',4'-Dihydroxy-6'-methoxy-3'-methylchalcone (5)

M.p. 198–203 °C. – UV:  $\lambda_{max}$  (MeOH) = 346.0 nm;  $\lambda_{\text{max}}$  (AlCl<sub>3</sub>, HCl) = 372.2 nm;  $\lambda_{\text{max}}$ (NaOMe) = 389.8 nm;  $\lambda_{max}$  (NaOAc, H<sub>3</sub>BO<sub>3</sub>) = 370.2 nm. – FT-IR (KBr):  $\nu_{\text{max}} = 3142, 2933, 2725,$ 1625, 1536, 1447, 1339, 1231, 1150, 1119, 976, 864, 795, 760, 699 cm<sup>-1</sup>. – <sup>1</sup>H NMR (300 MHz, C<sub>3</sub>D<sub>6</sub>O) [NOE, enhanced signal]:  $\delta = 2.00 (3H, s), 3.92 (3H, s)$ s) [6.15], 6.15 (1H, s) [3.92], 7.44 (3H, m), 7.71 (2H, m), 7.74 (1H, d, J = 15.6 Hz), 8.02 (1H, d, J =15.6 Hz), 14.51 (1H, s) [-]. The intensity of the signal at 14.51 decreased upon  $D_2O$  shake. – <sup>13</sup>C NMR (125 MHz, C<sub>3</sub>D<sub>6</sub>O) (DEPT) [HMQC] {HMBC, 400 MHz, CDCl<sub>3</sub>}:  $\delta = 7.36$  (CH<sub>3</sub>) [2.00], 56.1 (CH<sub>3</sub>) [3.90], 91.5 (CH) [6.15], 104.4 (C) {2.00, 6.15}, 106.0 (C) {6.15}, 128.6 (CH) [8.00], 129.0 (CH) [7.7], 129.0 (CH) [7.7], 129.7 (CH) [7.4], 129.7 (CH) [7.4], 130.7 (CH) [7.4], 136.4 (C), 161.8 (C) {2.00}, 163.3 (C) {3.92}, 166.4 (C) {2.00}, 193.1 (C). – EIMS (70 eV) m/z (rel. int. %) = 284.1  $(100) C_{17}H_{16}O_4$ , 267.1 (41.15), 256.1 (37.33), 207.1 (99.1), 181.1 (62.20), 165.0 (42.87), 151.1 (37.39), 122.0 (55.53), 103.1 (51.14), 77.0 (47.71).

## 2',4'-Dihydroxy-6'-methoxy-3',5'dimethylchalcone (**7**)

M.p.  $125-126 \,^{\circ}\text{C.} - \text{UV:} \lambda_{\text{max}} \text{ (MeOH)} =$ 335.8 nm;  $\lambda_{\text{max}}$  (AlCl<sub>3</sub>, HCl) = 368.8 nm;  $\lambda_{\text{max}}$ (NaOMe) = 409.8 nm;  $\lambda_{max}$  (NaOAc, H<sub>3</sub>BO<sub>3</sub>) = 421.4 nm. – FT-IR (KBr):  $\nu_{\text{max}} = 3335, 2945, 2860,$ 1629, 1548, 1424, 1359, 1312, 1231, 1169, 1115, 985, 911, 818, 760, 691 cm<sup>-1</sup>. – <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>) [NOE, enhanced signal]:  $\delta = 2.15$  (3H, s), 2.17 (3H, s), 3.66 (3H, s) [2.17, 8.00], 5.88 (1H, s), 7.41 (3H, m), 7.63 (2H, m), 7.84 (1H, d, J =15.7 Hz), 8.00 (1H, d, J = 15.7 Hz), 13.69 (1H, s). The intensity of the signals at 5.88 and 13.69 decreased upon D<sub>2</sub>O shake. - <sup>13</sup>C NMR (100 MHz,  $CDCl_3$  (DEPT) [HMQC] {HMBC}:  $\delta = 7.6$  (CH<sub>3</sub>) [2.17], 8.2 (CH<sub>3</sub>) [2.15], 62.3 (CH<sub>3</sub>) [3.66], 106.6 (C) {2.15, 13.69}, 109.0 (C) {2.15, 13.69}, 109.00 (C) {5.88}, 126.7 (CH) [8.00], 128.4 (CH) [7.41] {7.63}, 128.4 (CH) [7.41] {7.63}, 128.9 (CH) [7.63], 128.9 (CH) [7.63], 130.2 (CH) [7.41] {7.63}, 135.3 (C) {7.84}, 142.9 (CH) [7.84] {7.63, 8.00}, 158.8 (C) {3.66}, 159.3 (C) {2.17, 5.88}, 162.0 (C) {2.15, 13.69}, 193.4 (C)  $\{8.00\}$ . – EIMS (70 eV) m/z (rel. int. %) = 298.1 (100)  $C_{18}H_{18}O_4$ , 221.0 (93.12), 194.0 (80.67), 166.1 (24.11), 136.0 (20.47), 103.1 (33.04), 83.0 (49.84), 77.0 (21.92), 69.1 (14.18).

## Hydrogenation of compounds 5, 6 and 7

Hydrogenation (Laswell and Hufford, 1977) was done on a Parr hydrogenation apparatus model 3911 (shaker type hydrogenator) with 66CA 50 ml reaction bottle. Analytical grade acetone (for compound 5) or diethyl ether (for compounds 6 and 7) was used as solvent. Sample weighing 30 mg was placed in the reaction vessel together with 30 ml of solvent and 20 mg of catalyst, 10% palladium in carbon. Pressure was maintained at 30 psi and reaction time was 2 h. The mixture in the reaction bottle was filtered and the filtrate concentrated in vacuo. The crude product was chromatographed on silica gel and purified through a dropper column using isocratic elution with dichloromethane. Hydrogenation of compounds 5, 6 and 7 gave compounds 8 (12 mg), 10 (9 mg) and 11 (12 mg).

## Prolyl endopeptidase inhibition assay

Prolyl endopeptidase (*Flavobacterium meningo-septicum* origin) was purchased from Seikagaku Corporation (Tokyo, Japan). *N*-benzylocarbonyl-Gly-Pro-*p*NA and bacitracin were purchased from BACHEM Fine Chemicals Co. (Switzerland) and

Sigma Co., Ltd., respectively. PEP inhibitory activities were measured as described in Rahman et al. (2002). A solution of 255  $\mu$ l Tris HCl buffer (0.1 M, pH 7.0), 10 µl PEP (0.03 unit/300 ml) and test sample in  $8 \mu$ l MeOH to make 1 mM solution were mixed in a well of a 96-well microplate and preincubated for 5 min at 30 °C. The reaction was initiated by adding 30 µl of 2 mM of N-benzylocarbonyl-Gly-Pro-pNA (in 40% 1,4-dioxane). After incubation at 30 °C for 30 min, the amount of released *p*-nitroaniline was determined colorimetrically with the molecular device (96-wells microplate reader) at 410 nm. The concentrations of tested compound that inhibited the hydrolysis of substrate (N-benzylocarbonyl-Gly-Pro-pNA) by 50% (IC<sub>50</sub>) were determined by monitoring the effect of increasing concentrations of these compounds in the assay on the inhibition values. The  $IC_{50}$  values were then calculated using the EZ-Fit enzyme kinetics program (Perella Scientific Inc., Amherst, USA).

#### Thrombin inhibition assay

Thrombin inhibitory activities were measured as described by Anis *et al.* (2001). Enzymatic activity of bovine thrombin (Sigma) was measured in a buffer containing 0.145 M NaCl, 0.005 M KCl, 1 mg/ml polyethylene glycol (PEG-8000), 0.03 M 2-(4-(2-hydroxy-ethyl)-1-1-piperazinyl ethansulfonic acid (HEPES) (pH 7.4) and 0.1 U/well final concentration in the microtiter plate based assay. The enzyme was incubated with the inhibitor at 37 °C for 15 min before starting the reaction. The reaction

was initiated with the addition of 0.25 mM *N*-benzoyl-Phe-Val-Arg-*p*-nitroanilide. Time dependent optical density changes were followed at 405 nm by a 96-well microplate reader. The inhibition in percent was calculated by the following formula: % Inhibition =  $[(A-B)/A] \times 100$ , where A is the amount of *p*-nitroanilide liberated by the enzyme in the system without an inhibitor and B is the amount liberated with an inhibitor. Leupeptin was the standard used.

## Trypsin inhibition assay

Bovine pancreatic trypsin (Sigma) was assayed similar to thrombin, except that the buffer was 50 mm Tris-HCl (pH 7.5) and the reaction was started with 1 mm N- $\alpha$ -benzoyl-DL-Arg-p-nitro-anilide.

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